

Effects of Ionizing Radiation on Normal and Tumor-Associated Lymphatic Vessels

By

Jennifer D. Lobo

Submitted to the Department of Nuclear Science and Engineering

In partial fulfillment of the requirements for the degree of

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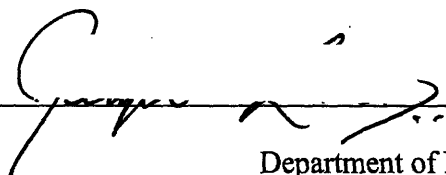
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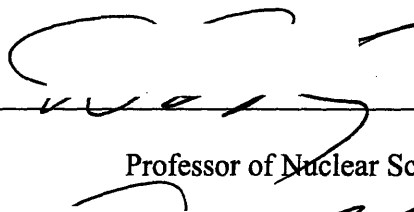


Jennifer D. Lobo

Department of Nuclear Science and Engineering

May 23, 2007

Certified By: _____



David G. Cory

Professor of Nuclear Science and Engineering

Thesis Supervisor

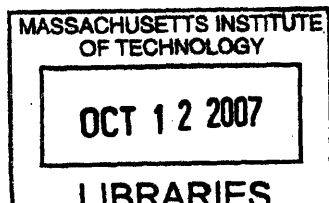
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David G. Cory

Professor of Nuclear Science and Engineering

Chairman, NSE Committee for Undergraduate Students



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ABSTRACT

Lymphatic vessels play a crucial role in both the pathophysiology of tumors and in the spread cancer cells to lymph nodes. The effects of radiation on these vessels, however, are largely unknown. Here, we seek to describe the effects of ionizing radiation on normal and tumor-associated lymphatic vessels in vitro and in vivo. Clonogenic assays were employed to study the radiation dose response of lymphatic endothelial cells. Putative lymphatic endothelial cell mitogens and antiproliferative agents, including vascular endothelial growth factor-A (VEGF-A), VEGF-C and AZD2171, a tyrosine kinase inhibitor of the VEGF receptors, were tested as radiation sensitizers and protectors. Our results indicate that VEGF-A and VEGF-C are radiosensitizers while AZD2171 did not modulate the radioresponse. In vivo, normal lymphatics were studied with the experimental group receiving a single fraction of 8 Gy and the control group receiving no radiation. We observed no difference in the average lymphatic vessel diameter between these two groups over the course of 6 months. VEGF-C overexpressing tumor-associated lymphatic vessels were studied in vivo with four treatment groups: control animals (no irradiation), 8 Gy two weeks prior to implantation, 8 Gy at the time of implantation and 16 Gy given in two fractions before implantation (two weeks prior to and at the time of implantation). The average lymphatic vessel diameter and frequency of lymph node metastasis in these four groups indicates that the ability of radiation to prevent VEGF-C driven lymph node metastases is time-dependent; radiation must be delivered in close proximity to VEGF-C overexpressing tumor cell implantation to impact nodal metastases. This suggests that VEGF-C may be a functional lymphatic vessel radiosensitizer in vivo. However, reductions in lymphatic hyperplasia, as measured by lymphatic vessel diameter, did not explain the observed differential effects of radiation timing on lymph node metastasis rate.

Thesis Supervisor: David G. Cory

Title: Professor of Nuclear Science and Engineering

Preface

Cancer is the second leading cause of death in the United States (1). Each year 10.9 million people worldwide are diagnosed with cancer accounting for 6.7 million deaths. It is estimated that there are 24.6 million people alive who have received a diagnosis of cancer in the last five years (2).

Cancer is generally treated by a combination of modalities: surgery, chemotherapy, and radiation. When able, surgeons remove the primary tumor, and chemotherapy and/or radiation are used to eradicate any remaining cancer cells. While all three modalities are commonly employed, over two-thirds of patients are treated with radiation, more than the number of patients treated with either surgery or chemotherapy (3). Because treating normal tissue is inevitable when treating cancer with radiation, the effects of radiation on normal and tumor-associated tissue are both of clinical interest. While adequate information on the effects of radiation on many types of cells and tissues is available (4), the effects of radiation on lymphatic vessels have largely been unreported. Lymphatic vessels play central roles in defining the tumor microenvironment during tumor progression and metastasis as well as governing the physiology of the post-treatment effects. For example, the absence of intratumoral lymphatic vessels contribute to the interstitial hypertension characteristic of solid tumors. Additionally, hyperplastic peritumoral lymphatic vessels may serve as a conduit for lymph node metastases. Furthermore, post-treatment, aberrant lymphovascular function contributes to lymphedema commonly associated with combined surgical and radiation therapy in an array of malignancies. This thesis will explore the effects of radiation on lymphatic endothelial cells based on data from carefully designed experiments.

Specific Aims

Aim 1: To determine the radiosensitivity of lymphatic endothelial cells.

Hypothesis 1: Lymphatic endothelial cells are more sensitive to radiation than blood vascular endothelial cells.

Rationale: The radiosensitivity of blood vascular endothelial cells has been documented. There is a paucity of data on the radiosensitivity of lymphatic endothelial cells (LECs). A more sensitive lymphatic endothelial population may explain the incidence of lymphedema after axillary lymph node dissection and radiation.

Aim 2: To determine whether lymphangiogenic growth factors can act as radiosensitizers or radioprotectors.

Hypothesis 2: VEGF-A and VEGF-C will decrease colony formation in a clonogenic survival assay.

Rationale: Rapidly-dividing cells spend more time in the mitotic phase of the cell cycle, a time when many cells are most prone to radiation induced death. Therefore, given the function of VEGF-A and VEGF-C to induce cell replication, we expect that these growth factors act as radiosensitizers. However, receptors for VEGF-A tend to be found on blood endothelial cells while the receptor for VEGF-C is found only on LECs. While both molecules should be radiosensitizers, we expect that VEGF-C will be more so than VEGF-A. Alternatively, both VEGF-A and -C provide a survival signal for LECs. In this context, VEGF-A and -C may act as radioprotectors.

Aim 3: To determine the efficacy of AZD2171, a VEGF family receptor inhibitor, as a radiosensitizer or radioprotector.

Hypothesis 3: AZD2171 will increase colony formation in a clonogenic survival assay.

Rationale: AZD2171, by blocking the major receptor pathways of lymphangiogenesis, will reduce the mitotic rate of the lymphatic endothelial cells and thus behave as a radioprotector.

Alternatively, AZD2171 may block important survival signals through the VEGF family, and thus act as a radiosensitizer.

In addition to resolving these aims, this thesis will also incorporate some *in vivo* data taken and analyzed in collaboration with Dr. Kevin Kozak. These data will broaden the context for the *in vitro* results and permit a thorough discussion of the role of the lymphatic system in cancer and its behavior after radiation exposure.

Introduction

The Lymphatic System

A critical role of the human lymphatic system is to maintain interstitial fluid homeostasis, that is, physiological equilibrium in the tissue space between physiological structures (e.g. cells and blood vessels). Lymphatic vessels drain fluid and macromolecules from the interstitium in order to keep the interstitial fluid pressure and the oncotic pressure constant (4).

Lymph is a clear fluid that originates from the blood plasma and contains various proteins and lymphocytes. Blood plasma leaks from capillaries into the body's tissues, permeates through tissue, and subsequently drains into the vessels of the lymphatic system, creating lymph. Lymph formation is generally governed by local convective flows at the vessel wall, which are dictated by the microarchitecture of the lymphatic endothelial cell (LEC). The lymphatic vessel wall consists of a highly attenuated endothelium, comprised of LECs, with a discontinuous basement membrane (4). Additionally, the absence of tight junctions between the LECs that comprise the vessel wall can create openings of several microns in the endothelial wall that allow free fluid motion between the tissue interstitium and the lymphatic vessel (4). Once within the lymphatic lumen, the lymph is transported by interstitium-induced peristalsis. Fluid flow within the lymphatic vessels of rabbit ears, for example, has been observed to be synchronized with arteriolar vasomotion (motion of blood vessels) (4). The motion of these blood vessels carries through the interstitium and causes movement along the neighboring lymphatic vessels. Larger lymphatic vessels do have a smooth muscle layer that allows active contraction to propel lymph, similar to the actions of veins in returning blood to the heart.

Key to the transport of lymph is the system of lymphatic valves. The majority of lymphatic valves are bicuspid, and to prevent retrograde flow, these valves must operate at very

low flow rates and in channels that may be of highly irregular geometry (4). Each valve consists of two collagen-based leaflets, each of which is attached to one side of the vessel wall.

Downstream, the leaflets converge. The pressure difference between each side of a given valve forces a separation between the leaflets and allows fluid flow through the valve. Once the fluid has passed through the valve, reverse motion of fluid is not observed if the leaflets are sufficiently flexible (4). The flow of fluid within the lymphatic system is an important transport mechanism for macromolecules and immune cells. The flow allows immune cells to gather in lymph nodes to maintain immunocompetency. In tumors, however, lymph flow is severely reduced due to compressive mechanical forces that collapse lymphatic vessels. As a result of the collapsed lymphatic vessels, tumors no longer maintain their fluid balance, resulting in a uniformly elevated interstitial fluid pressure. Elevated permeability of tumor blood vessels increases the volume of plasma that is leaked to the interstitium, which contributes to this rise in fluid pressure. The uniform fluid pressure restricts interstitial convection, which is the main mechanism of interstitial macromolecular transport. Therefore, molecular transport in tumors is restricted to the slower method of diffusion. The restricted fluid flow inhibits efficient and uniform delivery of chemotherapeutic drugs to the tumor for treatment (5).

In many cases, the failure of delivery of chemotherapeutic drugs to the tumor allows the cancer to advance and metastasize from the primary site to secondary sites in the body. Though cancer can spread hematogenously (through blood vasculature), lymphogenously (through lymphatic vessels) or by invasion of tissue adjacent to the tumor, lymphogenous metastasis is most common and cancer cell migration to the regional lymph nodes is a crucial step in the progression of cancer (6).

Additionally, lymphogenous tumor metastasis represents a central cause of cancer morbidity and mortality. Radiation treatment fields are often designed to encompass both the known tumor as well as draining lymphatics and lymph nodes (3). The therapeutic benefits of radiation treatment of lymphatic vessels include the sterilization of microscopic disease in regional lymphatics and lymph nodes. In essence, any metastatic cancer cells should undergo radiation-induced cell death following radiation treatment (7). However, an additional therapeutic effect of lymphatic vessel and nodal irradiation, namely a reduction in the efficacy of tumor cell spread through irradiated lymphatic vessels, has yet to be elucidated.

Although clearly effective and beneficial in the reduction of locoregional (i.e. lymph node) cancer recurrence, targeting lymphatics also increases potential morbidity, most notably, lymphedema (3). Radiation treatment of breast carcinoma, for example, has a low incidence of long-term complications; however, lymphedema is the most common of these and can profoundly detract from the quality of life of breast cancer patients. An elevated risk of lymphedema in patients who receive lymph vessel irradiation is clear (8). However, the mechanism responsible for this elevated risk remains elusive.

The mechanisms by which radiation therapy reduces lymph node recurrences and increases the risk of lymphedema require clarification, and an understanding of these mechanisms may contribute to the discovery and implementation of targeted prevention methods for lymphogenous tumor metastasis and lymphedema secondary to radiation therapy.

Growth Factors and Chemotherapeutic Agents

VEGF-A. Vascular endothelial growth factor-A (VEGF-A) is a pivotal stimulus of physiological and pathologic angiogenesis, including the continued formation of new blood

vessels to sustain solid tumor growth (9). These blood vessels provide tumors with oxygen and nutrients, in addition to providing routes for tumor metastasis. Angiogenesis is the product of a complex series of molecular events involving the activation of surrounding cells and changes in the microenvironment (10). The signaling induced by VEGF-A, however, is considered to be rate-limiting in this series of events. VEGF-A regulates several important processes of angiogenesis: endothelial cell migration and proliferation, recruitment of endothelial cell precursors, capillary tube formation, neovascular survival and enhanced vascular permeability, among others (11-17). Because so many of these processes are orchestrated by VEGF-A signaling, overexpression of VEGF-A will induce excessive angiogenesis. Such overexpression can be caused by oncogene activation (activation of a gene that induces cell replication) or loss of tumor suppressor function (deactivation of a gene meant to suppress cell replication) (18-20). The vessels resulting from overexpression of VEGF-A tend to be tortuous and dilated in comparison to vessels in normal tissue (21).

VEGF-A Signal Transduction Pathway. VEGF-A binds to the second and third immunoglobulin-like domains of two transmembrane receptors on endothelial cells: VEGFR-1 (Flt-1) and VEGFR-2 (KDR) (22, 23). This binding induces dimerization, which in turn causes conformational changes that stimulate kinase activity and transphosphorylation among tyrosine residues within the cell. These tyrosine residues serve as recognition sites for proteins that propagate intracellular signaling. Activated VEGFR-2 has specifically been shown to be the major stimulator of angiogenesis and vascular permeability (24, 25).

VEGF-C. Vascular endothelial growth factor-C (VEGF-C) is slightly more complicated than VEGF-A. VEGF-C mainly stimulates lymphangiogenesis, the formation of new lymphatic vessels and up-keep of the existing lymphatic vasculature system, and is a ligand of VEGFR-3,

which is primarily found on LECs in normal adults (26-30). In tumors, however, VEGFR-3 is upregulated and can be found on blood vessel endothelia as well (31, 32). Overexpression of VEGF-C is known to increase lymphangiogenesis and promote cancer metastases (33-35).

VEGF-C Signal Transduction Pathway. As a ligand of VEGFR-3, VEGF-C binds to VEGFR-3 and induces the autophosphorylation of tyrosine residues of the intracellular VEGFR-3 domain. This results in the binding of two signaling adaptor proteins, which in turn induce the binding of intracellular signal-related kinases. These signals promote endothelial cell proliferation, migration and survival (36-38). Stimulation of VEGFR-3, therefore, has been observed as a survival factor for lymphatic endothelial cells (39). Moreover, autophosphorylation of specific tyrosine kinases in this pathway induce a survival signaling cascade. It is of interest to this study to note that reactive oxygen species are also able to induce tyrosine phosphorylation of VEGFR-3, thereby promoting endothelial cell survival under redox stress (40).

Targetting the VEGF Family Pathways. Because VEGF-C is a known promoter of lymphangiogenesis and cancer metastases, we expect that targeting this pathway with chemotherapeutic agents would lead to fewer cases of metastatic cancer. Likewise, inhibiting the ability of VEGFR-2 to transduce a signal after binding with VEGF-A would inhibit angiogenesis and, in effect, starve the tumor and prevent its growth.

AZD2171. AZD2171, shown in Figure 1, is a highly potent (subnanomolar IC_{50}) tyrosine kinase inhibitor of VEGFR-2 and VEGF-induced signaling in endothelial cells (41).

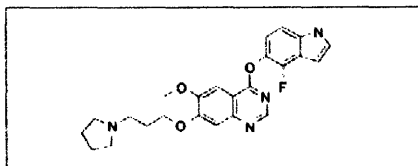
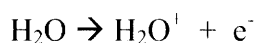


Figure 1. Structure of AZD2171. AZD2171 is a highly potent inhibitor of VEGFR-2 tyrosine kinase activity and VEGF-induced signaling in endothelial cells.

Given that angiogenesis does not occur in healthy adults (aside from wound healing and cyclical changes in the female reproductive tissues), inhibiting the VEGFR-2 signal transduction pathway may prove to inhibit tumor progression without interrupting most normal physiological processes (42).

Radiation and Mitotic Cell Death

Effects of Radiation on Cells. The biological effects of radiation result mainly from the damage done to DNA. This is true for any form of radiation, but for the purposes of this study, we will focus on radiations with low linear energy transfer (LET), specifically x-rays. Such low LET radiation tends to damage DNA through indirect action (43). As opposed to interacting with the target molecule itself, DNA, low LET radiations interact with other atoms or molecules in the cell to produce free radicals. Since water comprises 80% of a cell, the ionization of water from radiation is particularly relevant. When water interacts with an x-ray, the reaction



occurs, producing the H_2O^+ ion (43). This ion, in turn, reacts with another water molecule to produce the hydronium ion H_3O^+ and the highly reactive hydroxyl radical, OH^\cdot . This hydroxyl radical can diffuse, reach and react with the DNA and break the chemical bonds within this molecule, resulting in DNA damage. It is estimated that two-thirds of the x-ray damage to DNA in mammalian cells is due to the hydroxyl radical (43). Damaged DNA not only affects the cell's ability to fulfill its differentiated role and produce specific proteins, but, more importantly, it affects the cell's ability to replicate and the integrity of that replication. If a cell with damaged DNA still manages to replicate, the DNA of the daughter cells will be damaged, like that of the parent cell.

If DNA is the critical target, it is logical, then, for different stages of the cell cycle to have different degrees of sensitivity to radiation because the amount of DNA in the cell changes during the cell cycle. Cells are least sensitive to radiation during the synthesis (S) phase, presumably because cells' machinery for repairing DNA damage is most active and attainable during this phase, and they are most sensitive to radiation during mitosis (M phase) because the DNA has been replicated and the cell is dividing (43). Rapidly dividing cells spend more time in the M phase and are, therefore, more sensitive to radiation than cells that divide infrequently or not at all (43).

Reproductive Integrity. Using cell death as a measure of the effect of radiation on LECs requires a specific definition of cell death. Cell death can mean a loss of function (for differentiated cells) or a loss of reproductive capacity; for this study, we will use the latter. With this definition, it is possible, for example, for cells after radiation treatment to still be producing proteins, synthesizing DNA, and even going through a couple of cell divisions. However, if the cell has lost the capacity to divide indefinitely and produce a large number of progeny, the cell, by this definition, has lost its reproductive integrity and is dead (43). Cells that have survived and retained their reproductive integrity *in vitro* are said to be clonogenic. Cells that die do so mitotically, while attempting to divide, after irradiation.

Effects on Experimental Design. It is necessary for the experimental design to take into account that irradiation induces mitotic cell death *in vitro*. The *in vitro* experiments here are done with a clonogenic basis for this reason. Cells are plated in dishes, irradiated and incubated for two weeks, at which point they are stained, and the resulting colonies are counted. Cells that are mitotically dead may still divide during the two-week incubation period, but the colonies of the cells that truly survived the radiation are noticeably larger. A short incubation time of a few days,

for example, would not allow us to make this distinction since it is possible that even cells that have lost their reproductive integrity are still dividing in a few days' time.

Plating Efficiency and Survival Fractions. Survival fractions are determined based upon the number of cells plated and the number of colonies counted after the incubation period; however, because every cell that is plated does not necessarily adhere to the surface of the well, we must also take into account the plating efficiency of the cell line in use. The formula (Eqn. 1)

$$\text{Survival Fraction} = \frac{\text{Colonies Counted}}{\text{Cells Plated} * (\text{Plating Efficiency}/100)}$$

describes the relationship between the number of cells plated, the number of colonies counted, the survival fraction and the plating efficiency.

Experimental Design

We will accomplish the stated objectives and investigate our hypotheses by investigating the effects of radiation on normal LECs *in vitro*, normal lymphatic vessels *in vivo* and proliferating tumor-associated lymphatic vessels *in vivo*.

The LEC *in vitro* experiments were initially designed based on established experiments that have used human umbilical vein endothelial cells (due to functional similarity between these two types of cells) (41,44). We use classical radiobiological assays (e.g. clonogenic survival assays) to assess the radiation dose-response of these cells in the presence and absence of VEGF-A, VEGF-C, and AZD2171. In order to test the true nature of these molecules as radiosensitizers, two experiments must be done: adding the agent before the cells are irradiated, and adding the agent after the cells are irradiated. These conditions will distinguish between an agent that is a true radiosensitizer/radioprotector and an agent that induces lymphatic endothelial cell proliferation or death.

To study the dose response of normal lymphatic cells *in vivo*, we use the ear model in immunodeficient nude mice (45). Building on the *in vitro* experiments, we assess the temporal- and dose-response of lymphatic vessels to ionizing radiation. In the ear, we use *in vivo* fluorescent lymphangiography to characterize the structure and function of irradiated normal lymphatics and quantify the effects of radiation. This technique involves the injection of a fluorescent dye into the extracellular matrix of the area of interest. Only lymphatic vessels that drain fluid from the area of injection can be seen using this imaging technique; therefore, this method can determine the morphological characteristics of the functional lymphatics in that area but may not reveal every lymphatic vessel in the tissue.

The final aspect of this project focuses on the impact of ionizing radiation on tumor-associated lymphatic vessels *in vivo*. These experiments parallel the normal lymphatics *in vivo* experiments. We use *in vivo* fluorescent lymphangiography to characterize and quantify the lymphatic vessels after irradiation and subsequent tumor implantation in the ear.

For the *in vitro* experiments, clonogenic assays are our primary method of quantification, whereas for the *in vivo* experiments, the primary method is quantification of images from lymphangiographies performed at various time points.

Materials and Methods

Radiation Sources. Two different radiation sources are used in this study. A broad-field 250 kVp x-ray irradiator with a dose rate of 1.89 Gy/min was used for all the *in vitro* experiments (46). A ^{137}Cs isotropic gamma ray source, producing gamma rays of 1.176 MeV, was used for all the *in vivo* experiments (47).

Determination of LEC Plating Efficiency. LECs (Cambrex) were cultured at low passage in fully-supplemented endothelial cell media (Cambrex) and plated on fibronectin-coated 6-well plates to determine the plating efficiency. Additionally, feeder cells (lethally irradiated lymphatic endothelial cells) were used because of the standard use of feeder cells in HUVEC *in vitro* experiments. (LECs and HUVECs are functionally similar.) These non-viable feeder cells contribute proteins and nutrients to the growth medium of the viable cells and allow an equal number of cells to be plated in each well, even when the number of viable cells will be different. The ratio of viable cells to feeder cells was varied from well to well in the 6-well plate. A total of 4 plates were plated with the breakdown shown in Table 1.

Table 1. LEC Plating Efficiency Experiment.

Well	No. Viable Cells Plated	No. Feeder Cells Plated
A	50	4950
B	100	4900
C	300	4700
D	1000	4000
E	2500	2500
F	5000	0

The plates were incubated for 14 days after plating and subsequently stained with crystal violet. Colonies with at least 50 cells were counted, and the plating efficiency was determined using Eqn. 1. (The survival fraction was 1 because the cells were not exposed to radiation.)

Dose response of LECs. LECs were cultured at low passage in fully-supplemented endothelial cell media and plated on fibronectin-coated 6-well plates for this experiment. In order to yield a countable number of colonies, we used the plating efficiency calculated from the previous experiment and an assumed survival fraction. The dose response data of HUVECs provided the assumed survival fractions of the variation radiation doses (48). Table 2 shows the number of cells plated and the radiation dose administered for each plate. For all subsequently

described *in vitro* experiments, feeder cells were used. To avoid over-crowding the cells, a maximum of 20,000 cells was plated in each well. The difference between the number of cells plated and the maximum of 20,000 is the number of feeder cells plated for that well.

Table 2. Dose Response of LECs.

Plate	Wells	Dose (Gy)	Assumed Survival Fraction	Cells Plated
1	A, B, C	0	1	300
	D, E, F			600
2	A, B, C	2	0.33	800
	D, E, F			1600
3	A, B, C	4	0.05	5000
	D, E, F			10000
4	A, B, C	6	0.025	10000
	D, E, F			20000
5	A, B, C	8	0.002	20000
	D, E, F			20000

After the cells were counted, they were irradiated at the indicated dose in solution. The cells were then plated and incubated for 14 days. Colonies of at least 50 cells were counted after subsequent crystalviolet staining, and the survival fractions were determined according to Eqn. 1. (The plating efficiency was determined using the 0 Gy control.)

Clonogenic Assays with VEGF-A prior to Radiation. LECs were cultured at low passage in fully-supplemented endothelial cell media and plated on fibronectin-coated 6-well plates in starvation media according to Table 3.

Table 3. Plating of LECs for clonogenic assay with growth factors (VEGF-A and VEGF-C) prior to radiation.

Plate	Dose (Gy)	Cells Plated
1	0	600
2	0	1250
3	2	1600
4	2	3400
5	4	5400
6	4	11300
7	6	20000
8	6	20000
9	8	20000
10	8	20000

Starvation media was then added to wells A, B, and C while VEGF-A (Cambrex) in starvation media (at a concentration of 50 ng/ml) was added to wells D, E, and F. The plates were incubated overnight. The plates were then irradiated with the doses shown in Table 3, immediately washed with PBS, and incubated for 6 hours in starvation media. The cells were again washed with PBS and incubated in fully-supplemented media for 14 days with media being changed 7 days after the date of irradiation. After crystal violet staining, colonies were counted and survival fractions were determined using Eqn. 1.

Clonogenic Assays with VEGF-C prior to Radiation. LECs were cultured at low passage in fully-supplemented endothelial cell media and plated in fibronectin-coated 6-well plates in starvation media according to Table 3. Starvation media was then added to wells A, B, and C while VEGF-C (Cambrex) in starvation media (at a concentration of 500 ng/ml) was added to wells D, E, and F. The plates were incubated overnight. The plates were then irradiated with the doses shown in Table 4, immediately washed with PBS, and incubated for 6 hours in starvation media. The cells were again washed with PBS and incubated in fully-supplemented media for 14 days with media being changed 7 days after the date of irradiation. After crystal violet staining, colonies were counted and survival fractions were determined using Eqn. 1.

Clonogenic Assays with VEGF-A post Radiation. LECs were cultured at low passage in fully-supplemented endothelial cell media and plated on fibronectin-coated 6-well plates in starvation media according to Table 4.

Table 4. Plating of LECs for clonogenic assay with growth factors (VEGF-A and VEGF-C) post radiation.

Plate	Dose (Gy)	Cells Plated
1	0	400
2	0	800
3	2	1400
4	2	3000
5	4	6500

6	4	13600
7	6	14200
8	6	20000
9	8	20000
10	8	20000

After overnight incubation, the plates were irradiated at the doses shown in Table 4 and immediately washed with PBS. Starvation media was then added to wells A, B, and C while VEGF-A in starvation media (at a concentration of 50 ng/ml) was added to wells D, E, and F. Following an 6-hour incubation, the cells were washed with PBS and subsequently incubated for 14 days in fully-supplemented media with media being changed 7 days after the date of irradiation. After crystal violet staining, colonies were counted and survival fractions were determined using Eqn. 1.

Clonogenic Assays with VEGF-C post Radiation. LECs were cultured at low passage in fully-supplemented endothelial cell media on fibronectin-coated plates and plated in 6-well fibronectin-coated plates with feeder cells in starvation media according to Table 4. After overnight incubation, the plates were irradiated at the doses shown in Table 4 and immediately washed with PBS. Starvation media was then added to wells A, B, and C while VEGF-C in starvation media (at a concentration of 500 ng/ml) was added to wells D, E, and F. Following an 6-hour incubation, the cells were washed with PBS and subsequently incubated for 14 days in fully-supplemented media with media being changed 7 days after the date of irradiation. After crystal violet staining, colonies were counted and survival fractions were determined using Eqn. 1.

Clonogenic Assays with AZD2171 prior to radiation. LECs were cultured at low passage in fully-supplemented endothelial cell media, plated on fibronectin-coated 6-well plates according to Table 5, and allowed to adhere overnight.

Table 5. Plating of LECs for clonogenic assay with AZD2171 prior to radiation.

Plate	Dose (Gy)	Assumed Survival Fraction	Cells Plated
1	0	1	400
2	0	1	700
3	2	0.436	900
4	2	0.436	1600
5	4	0.09	4400
6	4	0.09	7700
7	6	0.027	14800
8	6	0.027	20000
9	8	0.0059	20000
10	8	0.0059	20000

The cells were then incubated in concentrations of 0 nM, 2 nM, and 20 nM AZD2171 in starvation media (no growth factors) for 4 hours. For all plates, wells A and B were incubated with 0 nM, wells C and D with 2 nM, and wells E and F with 20 nM. The plates were irradiated according to Table 5 and subsequently incubated for 1 hour. The AZD2171-infused media was then aspirated. The cells were washed with PBS and incubated for 4 hours in starvation media. This media was then aspirated and replaced with fully-supplemented media. The cells were incubated for 14 days with the media being changed after 7 days and then stained with crystal violet. Colonies were counted and survival fractions were determined using Eqn 1.

Clonogenic Assays with AZD2171 post radiation. LECs were cultured at low passage in fully-supplemented endothelial cell media, plated on fibronectin-coated 6-well plates according to Table 5, and allowed to adhere overnight. The media was then aspirated and the cells were incubated in starvation media for 4 hours. The plates were irradiated according to Table 5 and then incubated for 1 hour. The media was aspirated and the cells were incubated in concentrations of 0 nM, 2 nM, and 20 nM AZD2171 in starvation media for 4 hours. For all plates, wells A and B were incubated with 0 nM, wells C and D with 2 nM, and wells E and F with 20 nM. The AZD2171-infused starvation media was aspirated. The wells were washed with PBS, replaced with fully-supplemented media, and allowed to incubate for 14 days with the

media being changed 7 days after the date of irradiation. After crystal violet staining, colonies were counted and survival fractions were determined using Eqn. 1.

In vivo dose response of normal lymphatics. Two groups of 20 immunodeficient nude mice were initially established. Following anesthesia, a dose of 8 Gy was given to the right ear of each mouse in the experimental group using a custom designed irradiation chamber and the previously described ^{137}Cs source. To control for the impact of anesthesia and placement in the irradiation chamber, mice in the control group were treated identically except that the irradiation chambers were not placed within the irradiator. Lymphangiographies have been done at 2, 4, 8, and 26 weeks after the irradiation. The 52-week time point will occur in July 2007. The results for the first four time points have been quantified, and the ears will be excised for immunohistochemical analysis.

In vivo dose response of tumor-associated lymphatics. Four groups of 20 immunodeficient nude mice were required for these experiments. Three experimental groups were employed to discern time- and dose-dependent radiation effects on tumor associated lymphatics. Mice were subjected to ear irradiation at dose of 0 Gy (control), 8 Gy (either two weeks prior to tumor implantation or at the time of tumor implantation), and 16 Gy (two fractions of 8 Gy delivered two weeks prior to and at the time of tumor implantation). We then implanted 100 μl of a single-cell suspension of T241 fibrosarcoma cells overexpressing VEGF-C into the irradiated ear [10]. Tumors were grown to 60-80 mm^3 and fluorescent lymphangiography was conducted. Following lymphangiography, tumors were excised and embedded in paraffin for immunohistochemical analysis. Cervical lymph node metastases were quantified as a functional endpoint.

Results

Plating Efficiency of LECs. Table 6 shows the number of colonies counted and the plating efficiencies obtained from this experiment.

Table 6. Plating Efficiency of Lymphatic Endothelial Cells.

Well Number	No. Colonies	Plating Efficiency
1A	3	0.06
1B	15	0.15
1C	18	0.06
1D	49	0.049
2A	3	0.06
2B	2	0.02
2C	18	0.06
2D	40	0.04
3A	5	0.1
3B	12	0.12
3C	26	0.086666667
3D	47	0.047
4A	8	0.16
4B	11	0.11
4C	13	0.043333333
4D	46	0.046

The number of colonies observed in wells E and F for all plates could not reasonably be counted. These data points were therefore dropped, and a plating efficiency of $7.6 \pm 1.0\%$ was calculated based upon the data from the other four wells. Because the calculated plating efficiency was low, we continued to use feeder cells in subsequent experiments with LECs.

Dose response of LECs. Figure 2 shows the results of the three repetitions of this experiment.

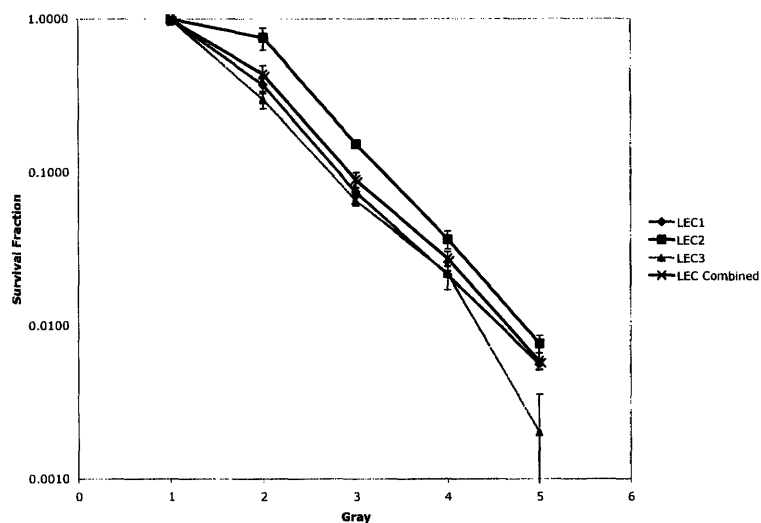


Figure 2. Radiation dose response of LECs.

Averaging the results of these three experiments produces the combined curve. We were therefore able to determine that the survival fraction at 0 Gy is 1.00 ± 0.037 , at 2 Gy is 0.44 ± 0.058 , at 4 Gy is 0.090 ± 0.010 , at 6 Gy is 0.027 ± 0.0030 , and at 8 Gy is 0.0059 ± 0.00075 .

Clonogenic Assays with VEGF-A and Radiation. Figure 3 shows the result of incubating cells with VEGF-A prior to radiation treatment while Figure 4 shows the result with VEGF-A incubation after radiation treatment.

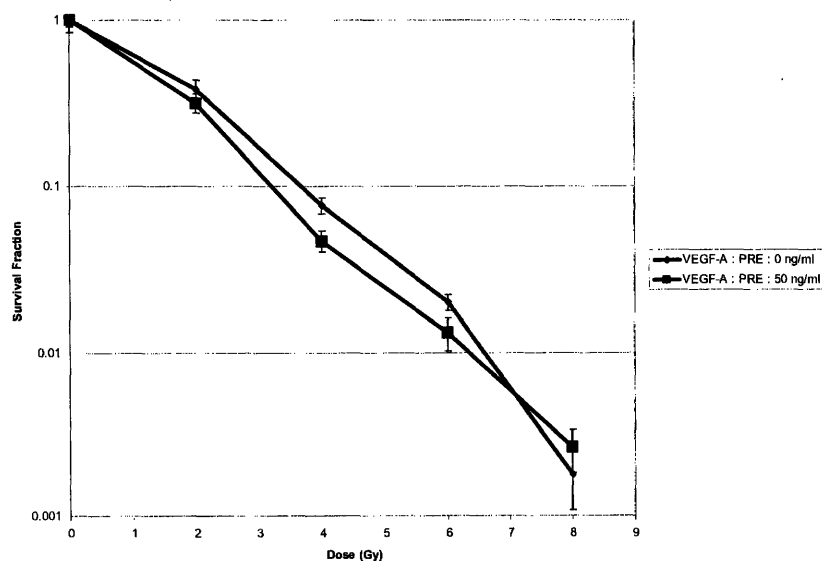


Figure 3. LEC dose response with VEGF-A prior to radiation

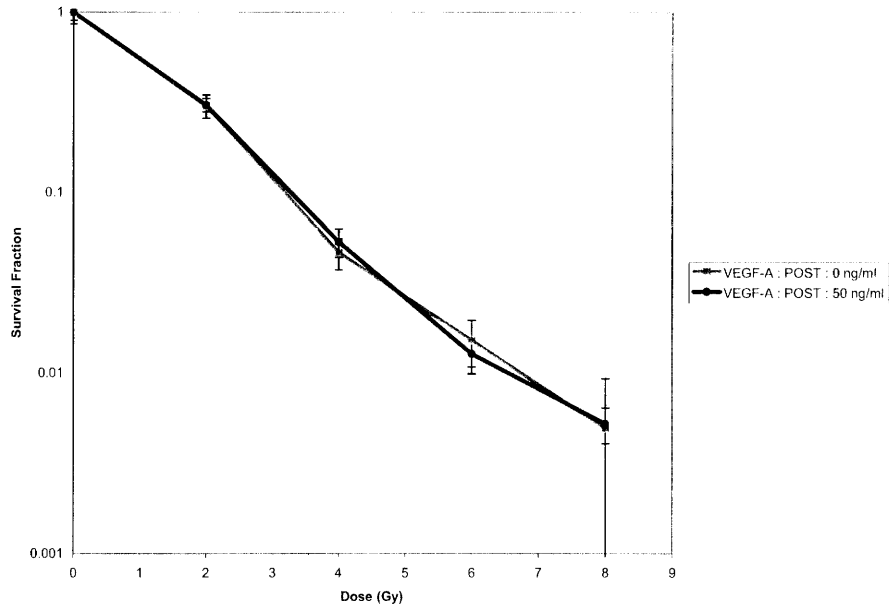


Figure 4. LEC dose response with VEGF-A post radiation

Clonogenic Assays with VEGF-C and Radiation. Figure 5 shows the result of incubating cells with VEGF-C prior to radiation treatment while Figure 6 shows the result with VEGF-C incubation after radiation treatment.

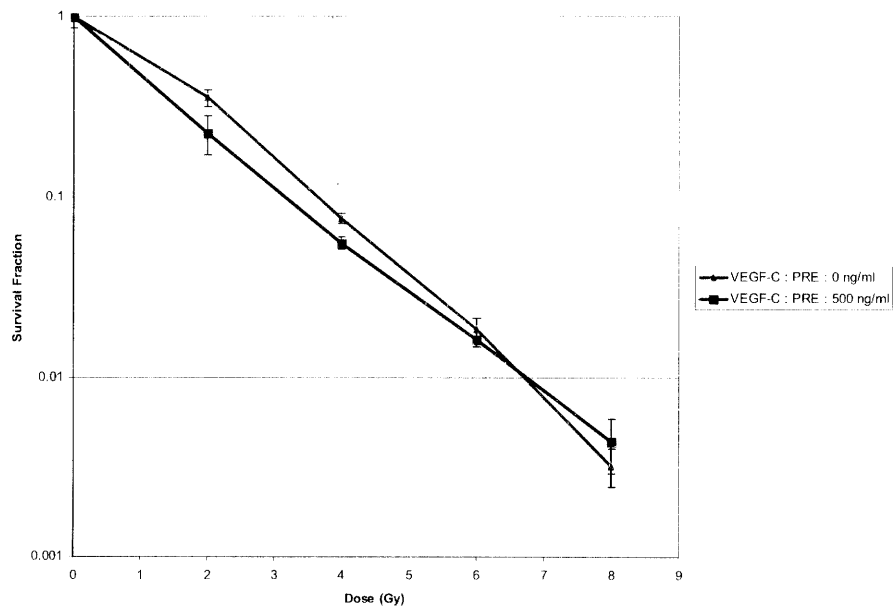


Figure 5. LEC dose response with VEGF-C prior to radiation.

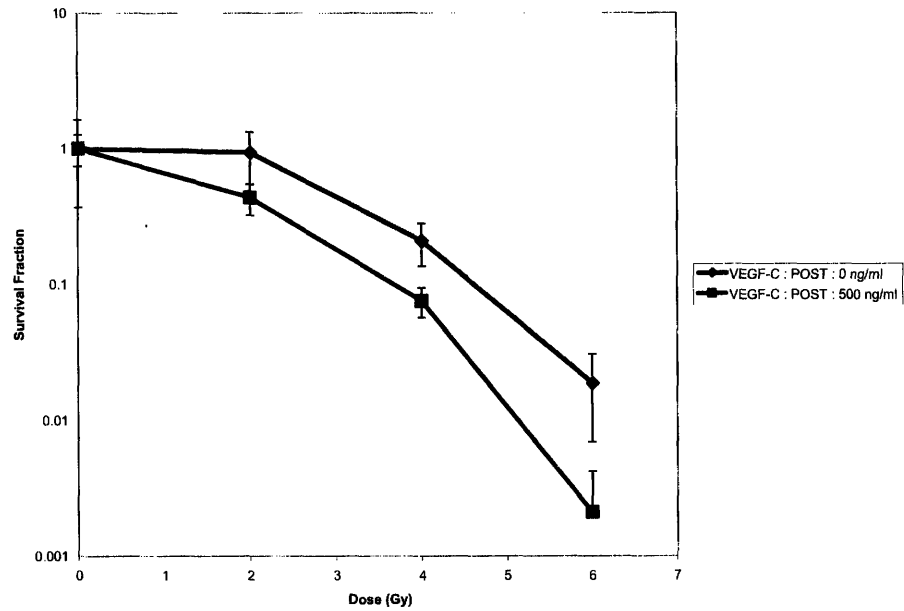


Figure 6. LEC dose response with VEGF-C post radiation.

Clonogenic Assays with AZD2171 and Radiation. Figure 7 shows the result of incubating cells with AZD2171 prior to radiation treatment while Figure 8 shows the result with AZD2171 incubation after radiation treatment.

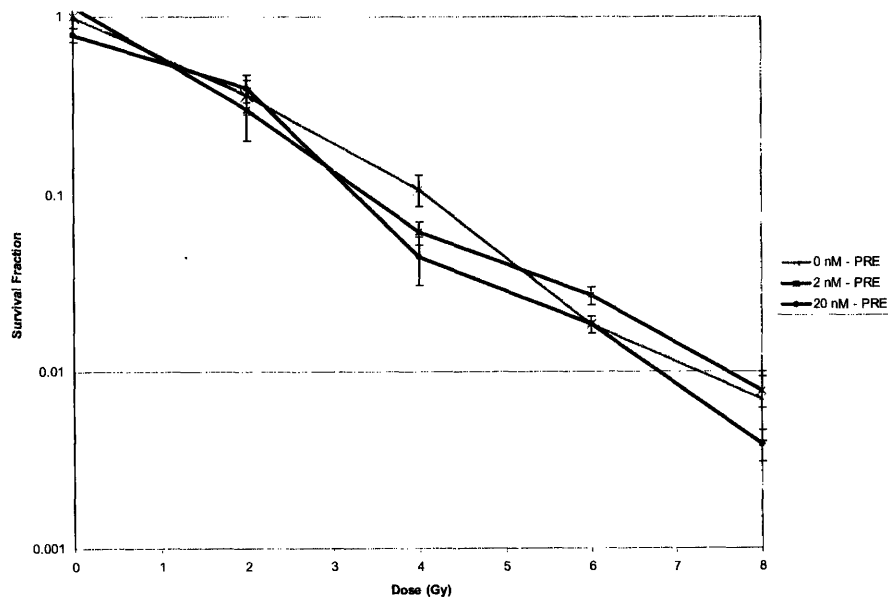


Figure 7. LEC dose response with concentrations of AZD2171 prior to radiation

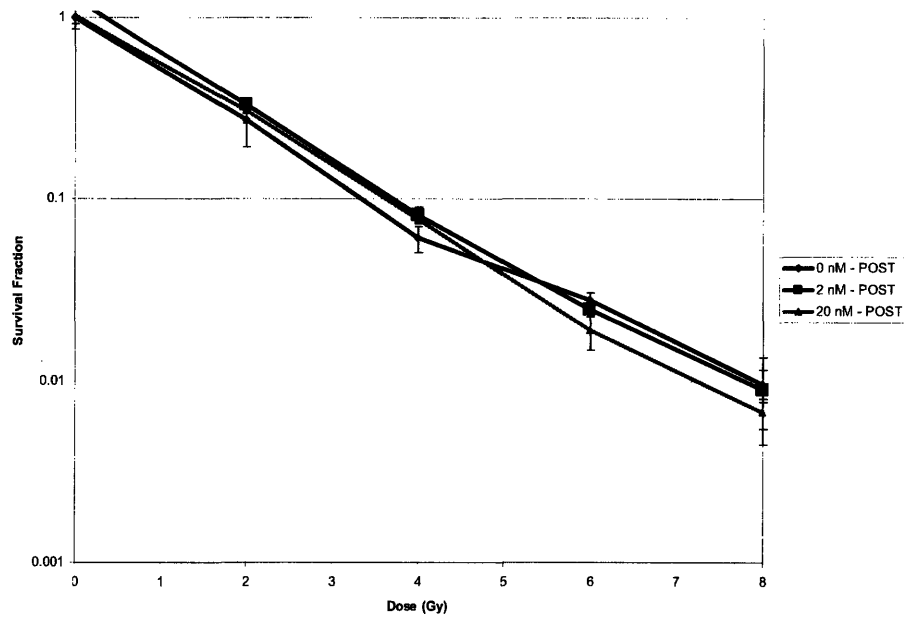


Figure 8. LEC dose response with concentrations of AZD2171 post radiation

***In vivo* dose response of normal lymphatics.** Figure 9 reports the average lymphatic vessel diameter for the two groups in the normal lymphatic *in vivo* experiment.

Figure 9. Lymphatic vessel diameter in normal mouse ear with and without radiation.

***In vivo* dose response of tumor-associated lymphatics.** The average vessel diameter for each group as reported from the lymphangiograms is shown in Figure 10.

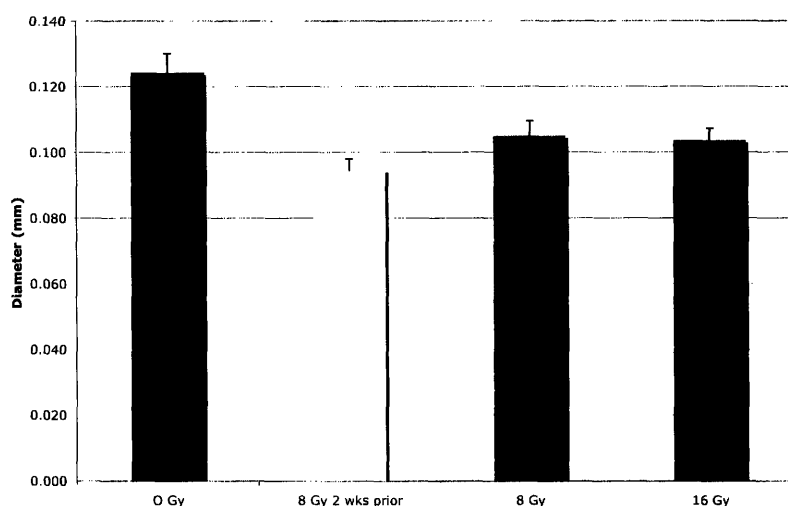


Figure 10. Average lymphatic vessel diameter for groups in tumor-bearing mice experiment.

Additionally, the size of the tumor and the occurrence of lymph node metastasis were recorded for each mouse in each of the four groups. The results are shown in Table 7.

Table 7. Average Tumor Size and Occurrence of Lymph Node Metastasis in Tumor-Bearing Mice.

	0 Gy	8 Gy - 2wks prior	8 Gy	16 Gy
Lymph Node Metastasis	15/20 (75%)	14/17 (82%)	7/18 (39%)	3/15 (20%)
Average Tumor Volume (mm ³)	71.9 ± 2.63	78.2 ± 4.84	70.3 ± 3.71	72.3 ± 3.57

Discussion

In Vitro Experiments

The results of the in vitro experiments show that LECs are radiosensitive, as evidenced by the lack of a shoulder in the dose response curve in Figure 2. The lack of a shoulder region in the curve demonstrates that radiation reduced mitotic capacity even at the lowest levels of radiation.

We next test the dose response of LECs with VEGF-A, VEGF-C and AZD2171 before and after radiation.. In order to test the true nature of these molecules as radiosensitizers, two experiments had to be done: adding the agent before the cells are irradiated, and adding the agent

after the cells are irradiated. Potentially lethal DNA damage that would cause cell death under normal circumstances can be repaired and the fraction of cells surviving a given dose of x-rays is enhanced if post-irradiation conditions are suboptimal for growth (43). Suboptimal conditions prohibit cells from attempting the complex process of mitosis while their chromosomes are damaged. If mitosis is delayed by suboptimal growth conditions, DNA repair can occur (43). Irradiating in the presence of a molecule and observing that the cells were unable to repair the damage indicates that the agent is a radiosensitizer. A true sensitizing agent must be present during the irradiation. Adding a molecule to the cells immediately following irradiating and observing that the cells were unable to repair the damage and maintain clonogenicity confirms that the agent is not a radioprotector. This pair of experiments, therefore, was designed to test whether the agent is a true sensitizer.

As was expected, VEGF-A and VEGF-C are radiosensitizers. This is evident in Figures 3 and 5 for the radiation doses of 2, 4, and 6 Gy. That these growth factors appear to be radiosensitizers in Figures 3 and 5 but not in Figures 4 and 6 demonstrates the need for these factors to be present during the irradiation in order to be radiosensitizing. The results of the AZD2171 experiments, on the other hand, indicate that this chemotherapeutic agent is not a radiosensitizer. The curves in Figure 7 fail to follow a general trend as they intersect between various doses, and Figure 8 shows virtually no difference in the dose response of LECs when AZD2171 is present after the irradiation. While these results do not prove that AZD2171 imparts any radioresistance for these cells, they indicate that no radiosensitivity is imparted either. Figures 3 and 5 are of concern to the experimental design as the curves in these figures intersect between 6 and 8 Gy. While this cross is somewhat perplexing given the trend set by the lower doses, this result is likely due to the experimental design. For all the in vitro experiments, a

maximum of 20,000 cells was plated in each well to avoid overcrowding the cells even though, ideally, tens of thousands of more cells should have been plated to achieve our target number of colonies (based upon the plating efficiency and the expected survival fraction). Because of this experimental limitation, it is reasonable to conclude that the results at 8 Gy lack integrity because of the low total number of colonies counted at 8 Gy.

Also of experimental note is the nature of the irradiation for these experiments. The dose response of LECs experiment was done three times. For the first two repetitions, the cells were irradiated in solution and then plated. For the third repetition, the cells were irradiated in the plate. That we saw no significant difference in the survival fractions indicates that irradiating in solution versus in the plate did not appreciably affect our results.

In Vivo Experiments

Figure 9 shows the results of the normal lymphatic vessel experiments; we observed no difference in the average lymphatic vessel diameter between the 0 Gy and 8 Gy groups. This seems to indicate that gamma radiation has no affect on the lymphatic system in normal tissue. However, while the vessel diameter may have remained the same, the number of functional vessels may have changed. In these normal tissue groups, there is no tumor acting as an organ to induce lymphangiogenesis. Thus, this experiment tests the affect of radiation on existing vessels. Therefore, it is unlikely that we would observe a narrowing of the diameter in vessels that are already functional parts of the normal lymphatic vasculature. The lymphangiography technique measures only functional vessels, making it difficult to determine the number of existing but dys- or non-functional vessels. Total lymphatic density can be determined immunohistochemically using LYVE-1 as a marker for lymphatics. Alternatively, we could quantify the edema, swelling

which results from fluid build up presumably because of dysfunctional lymphatic vessels. Quantifying edema is challenging as an established technique to accomplish this does not exist. However, there is evidence that magnetization transfer can be performed to quantify vasogenic edema in patients with multiple sclerosis (49), implying the possibility of applying this technique to tumor-induced lymphedema.

Figure 10 summarizes the results of the *in vivo* experiments studying the effects of gamma radiation on tumor-bearing mice, and Table 7 shows the average tumor size and the frequency of lymph node metastasis for each of these groups. Radiation clearly has an effect on the lymphatic vessel diameter as evidenced by the difference shown in Figure 10 between the 0 Gy group (Group 1) and the remaining groups, 8 Gy two weeks prior to tumor implantation (Group 2), 8 Gy at the time of tumor implantation (Group 3), and 8 Gy x 2 (16 Gy) (Group 4). The difference between Groups 2 and 3 in Figure 10 can be attributed to the growth of a more robust tumor in Group 2. While the tumor was implanted immediately after the damage by radiation was done to the tissue in Group 3, the tissue in Group 2 had two weeks to recover from the radiation damage. Implanting a tumor in a recovered environment versus an acutely damaged one would presumably better enable the tumor to grow, make use of its microenvironment and initiate angiogenesis and lymphangiogenesis. The low vessel diameter of Group 2 reported in Figure 10 suggests that the radiation may have weakened the lymphatic system.

The lack of significant difference between Groups 3 and 4 despite the significant difference in dose indicates the importance of the time of irradiation relative to the time of tumor implantation. The relatively equal vessel diameter between these two groups, in addition to the equal tumor sizes and the lymph node metastasis rates being relatively similar, imply that the difference of 8 Gy of radiation given two weeks prior did not significantly affect the lymphatic

system. This is most likely due to the two week recovery time between the fractions. It seems that this two week period was enough time for the tissue to recover to its normal state such that the 8 Gy fraction at the time of tumor implantation affected Group 4 just as it did Group 3. The difference between Groups 2 and 4 further illustrates the significance of the fraction given at the time of tumor implantation. The two week period between irradiation and tumor implantation in Group 2 allowed the tissue to recover back to a normal state such that the tumor was implanted in an ideal environment for its healthy growth. The recovery that likely occurred during the two week period between fractions in Group 4, however, was discounted by the second 8 Gy fraction at the time of tumor implantation. Like those in Group 3, the Group 4 tumors were less able to induce their own lymphatic vessels. Additionally, the microenvironment had been weakened by the fraction at the time of tumor implantation, compromising not only the angiogenic system which led to less healthy tumors and the decreased tumor size in comparison to Group 2, but also the function of the lymphatic vessels, resulting in a lower frequency of lymph node metastasis.

Future Experiments

The results and interpretations presented here lead to several relevant questions than can be answered with experiments done as an extension of those described here. This study has brought to light three main areas for continued focus regarding future experiments studying the effect of gamma radiation on normal and tumor-associated lymphatic vessels: lymphatic vessel repair, lymphedema and metastasis.

Because the function of lymphatic vessels is critical to the transport of fluid and cells, and thus the development of edema and metastasis, the time needed to restore function in these

vessels is important, especially in the context of fractionated therapy. While literature on cell survival is available for various cell lines in vitro (4), this literature is not particular to LECs and is not addressed in a relevant in vivo model. Investigating the vessel repair time will give us an idea of what the critical times points are for the development of edema and metastasis, perhaps enabling us to deliver radiation fractions in a manner that avoids these critical time points. Understanding the effect of fractionation on cell survival can be done by modifying our clonogenic assay.

As lymphedema is a chronic problem with patients who undergo radiation treatment (3), finding a way to minimize edema and improve the patients' quality of life is a worthy endeavor. Ideally, with experimentation, we would find a time period between fractions that allows the lymphatic vasculature to recover from the radiation and, therefore, minimizes lymphedema but does not allow the lymph system to become healthy enough that the chance of metastasis increases.

Metastasis, in many circumstances, is considered an event that deems whether a patient's cancer is curable; as such, understanding the state of the lymphatic system when metastasis is most likely to occur is of critical importance. Additionally, the effect of hypoxia on an irradiated lymphatic system and on metastasis is of interest. Many cancers metastasize in the late stages of development, when the tumor may be of considerable size. The tortuous nature of tumor blood vasculature makes delivery of blood, and therefore oxygen, less efficient than in normal tissue (5). Thus, in large tumors, areas of hypoxic tissues, which become necrotic due to lack of oxygen and nutrients, are observed, especially towards the center of the tumor (5). The presence of oxygen in tissue greatly increases the efficacy of radiation treatment. X-rays create oxygen free radicals which, as a highly reactive species, are able to commit considerable damage to cells and

their DNA. Even though only a small amount of oxygen is needed to produce this dramatic and important oxygen effect with x-rays, the magnitude of the effect increases with the concentration of oxygen (43). The effect of reoxygenation must also be considered here: during radiation treatment, hypoxic cells move to aerated regions of the tumor. If this effect is efficient, then the presence of hypoxic cells will not have a significant effect on the outcome of a multifraction regimen (43). Still, if late-stage tumors tend to metastasize more frequently and if these tumors are also hypoxic and are treated with radiation, then the mechanism and timeline of metastasis under these circumstances remains unclear.

Experimentally, we can address these three points of interest with one model experiment. The basic model would involve administering x-ray fractions after the implantation of GFP tumor cells in the mouse ear and observing the state of the lymphatic system by tracking the movement of the GFP tumor cells through the lymphatic vasculature, by lymphangiography to observe the number of functional vessels and their size, and possibly by magnetization transfer in an effort to quantify the lymphedema that is likely to result from the treatment.

The main variables to be tested would include time intervals between fractions and the amount of oxygen present in the tumor. Experimenting with the time between fractions while assessing the lymphatic system using the techniques mentioned above would allow us to place the development of lymphedema and metastasis in a time frame context. These techniques would help to describe the lymphatic system at the specific time when these events do develop such that certain characteristics observed under these circumstances but not under normal circumstances could be targeted in order to prevent edema and metastasis. Tracking the GFP tumor cells would especially be helpful in pinpointing when these cells start to spread to other regions of the body, and at these time points, lymphangiography could be done to characterize the lymphatic system

when this specific event is observed. In addition to lymphangiography and tracking when these cells metastasize, quantifying the edema at various time points would also contribute to our understanding of lymphatic vessel repair.

The two key events that the lymphatic system in tumors is linked to are the development of lymphedema post-radiation and treatment and the occurrence of metastasis. The goal of experiments extending from this study, therefore, would ideally address and pursue an understanding of these events and what, biologically, contributes to their successful occurrence. Studying the lymphatic vasculature in tumors as it changes through radiation response and recovery (vessel repair), edema, and metastasis and using the techniques of lymphangiography, magnetization transfer (quantifying edema), and GFP tumor cell tracking would contribute to our understanding of these events.

Conclusion

The effects of gamma radiation on normal and tumor-associated lymphatic vessels were studied in vitro and in vivo. The radiation dose response of lymphatic endothelial cells with the presence of VEGF-A, VEGF-C and AZD2171 both before and after irradiation was observed and has allowed us to characterize VEGF-A and VEGF-C as radiosensitizers. Our results do not indicate that AZD2171 is a radiosensitizer. In vivo, no difference between the control and irradiated groups on mature ear lymphatic vessels was observed. . The reported lymphatic vessel diameter, tumor size, and frequency of lymph node metastasis in the four different tumor-bearing groups indicates that a two-week time period between irradiation and tumor implantation allows a healthier tumor to develop while irradiation at the time of tumor implantation weakens the tumor's microenvironment and stunts the tumor's ability to grow and metastasize. These results imply that radiation affects the function of lymphatic vessels and that these vessels are more radiosensitive in the presence of over-expressed growth factors. That different results were observed for the four tumor-bearing groups indicates that the specific state of the lymphatic system will lead to certain outcomes regarding edema and metastasis. Future experiments would ideally address and characterize these specific states in hopes of targeting the tumor-associated abnormalities in the lymphatic system that lead to the development of lymphedema and metastasis.

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